METHODS AND REVIEWS

Associates and sponsors are encouraged to share ideas, applications research, applications experience, and technical problems by contributing to this column. In addition, short reviews covering aspects of biotechnology that are of interest to members are also encouraged. All contributions will be peer-reviewed, and the deadline for submitting articles for the September issue is August 1, 1997. Periodically articles will be solicited from non-members by the Editorial Board. Contributions may be mailed or faxed to the Editor, Clayton Naeve, Center for Biotechnology, St. Jude Children's Research Hospital, 332 N. Lauderdale St., Memphis, TN 38105-2794, Fax: (901) 495-2945, E-mail: clayton.naeve@stjude.org. Contributions to this column do not necessarily reflect the views of the Editorial Board or the Association.

MATRIX ASSISTED LASER DESORPTION IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY

John J. Lennon
University of Washington

Time-of-Flight Mass Spectrometry Principles

Linear TOF-MS With the increase in interdisciplinary research in recent years, the need for accurate and sensitive methods for the analysis of biomolecules has been increasingly important for both the chemist and biologist. Mass spectrometry has emerged as an important tool for analyzing and characterizing large biomolecules of varying complexity. The matrix assisted laser desorption/ionization (MALDI) technique (1), developed in 1987, has increased the upper mass limit for mass spectrometric analyses of biomolecules to over 300,000 Da and has enabled the analyses of large biomolecules by mass spectrometry to become easier and more sensitive. An attractive feature of the time-of-flight (TOF) mass spectrometer is its simple instrumental design (Figure 1). TOF mass spectrometers operate on the principle that when a temporally and spacially well defined group of ions of differing mass/charge (m/z) ratios are subjected to the same applied electric field (K.E. = $[mv^2]/2$ = zeEs where K.E. = kinetic energy; m = the mass of the ion; v = velocity of the ion; z = number of charges; e = the charge on an electron in coulombs; E = electric field gradient; and s = the distance of the ion source region) and allowed to drift in a region of constant electric field, they will traverse this region in a time which depends upon their m/z ratios.

Reflectron TOF-MS Improved mass resolution in MALDI TOF-MS has been obtained by the utilization of a single-stage or a dual-stage reflectron (RETOF-MS). The reflectron, located at

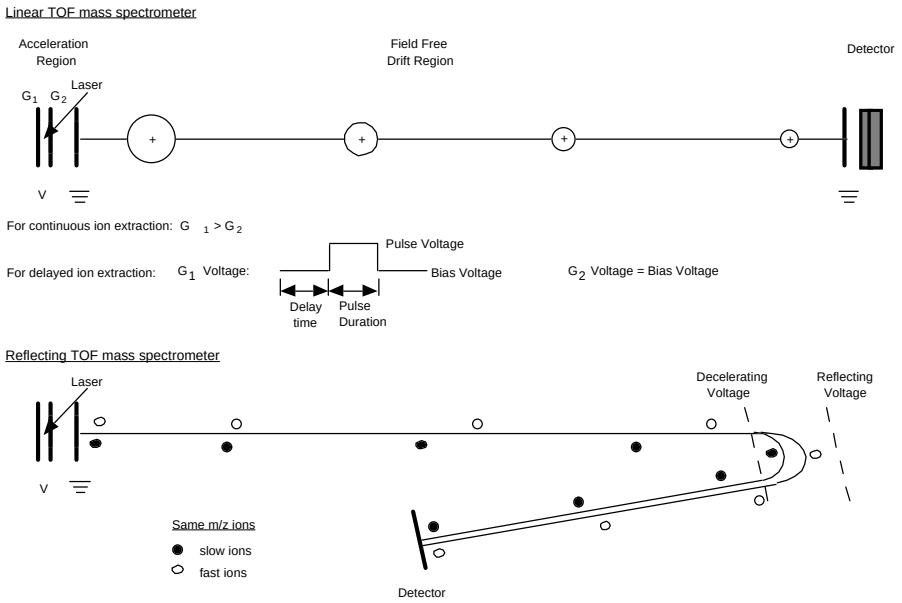

Figure 1. Basic components of a linear (upper panel) and reflecting (lower panel TOF mass spectrometer.

the end of the flight tube, is used to compensate for the difference in flight times of the same m/z ions of slightly different kinetic energies by means of an ion reflector (Figure 1). This results in focusing the ion packets in space and time at the detector. A typical MALDI mass spectrum of substance P in CHCA (see Table 1) employing both linear and reflectron TOF-MS in the continuous ion extraction mode with a 500 MS/s transient digitizer is shown in Figure 2. The maximum mass resolution observed in the linear mass spectrum of substance P employing continuous ion extraction is about 600 which is typical for a peptide of this size. Only the average chemical mass can be determined from this mass spectrum. In the reflectron mass spectrum, the isotopic multiplet is well resolved producing a full width half maximum (FWHM) mass resolution of about 3400. Mass resolutions up to 6000 (FWHM) have been obtained for peptides up to about 3000 Da with RETOF-MS (2). Enhancing the mass resolution can also increase the mass accuracy when determining the ion's mass.

Delayed Ion Extraction Linear TOF-MS The primary contribution to mass resolution loss in conventional (i.e., continuous ion extraction) linear MALDI TOF-MS is attributed to a range of flight times of identical m/z ions due to different initial velocities. A fraction of the final velocity that is attained for particular m/z ions as they are accelerated out of the ion source and into the field free region includes this initial velocity component. No compensation is made with continuous ion extraction linear TOF-MS for ions with the same m/z but different initial ion velocities. Improvements in mass resolution can be achieved by utilizing delayed pulsed ion extraction (3,4) [DE] as shown in Figures 1

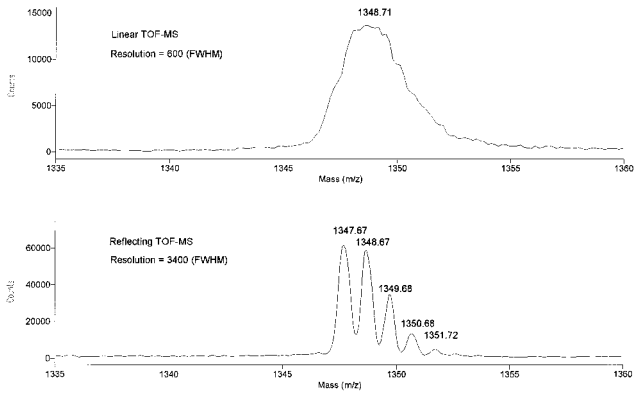

Figure 2. MALDI mass spectra of substance P in CHCA comparing mass resolution obtained in the linear (upper) and reflecting (lower) modes utilizing continuous ion extraction at threshold laser irradiance. 25 kV accelerating potential, 50 laser pulses averaged.

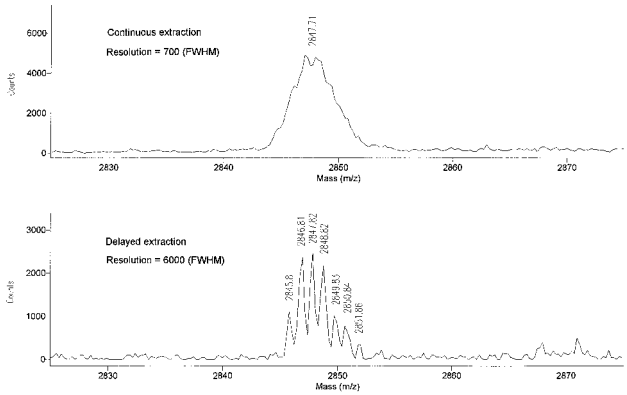

Figure 3. MALDI mass spectra of melittin in CHCA comparing mass resolution obtained in the linear mode utilizing continuous ion extraction (upper) and delayed ion extraction (lower) at threshold laser irradiance. 25 kV accelerating potential, 50 laser pulses averaged.

and 3 which can compensate for the initial velocity distribution of the MALDI generated ion packet such that same m/z ions arrive simultaneously at a space focal plane located at the detector. Broadening of the ion velocity distribution due to collisional processes in the ion source can also be minimized by allowing the dense plume of MALDI generated ions/neutrals to dissipate prior to ion drawout from the ion source. This results in narrower ion arrival time distributions and provides better mass resolution when compared to continuous ion extraction. Figure 3 shows the improved mass resolution obtainable with DE (and a 500 MS/s transient digitizer) for melittin. A systematic study employing DE (3) can give insight into the specific relationships/trends between such variables as the pulsed extraction voltage amplitude employed, an ion's m/z value, and the pulsed extraction delay time in order to obtain maximum mass resolution. All of the mass spectral data shown was collected using a PerSeptive Voyager Elite TOF mass spectrometer.

MALDI Sample Preparation

Typical MALDI Sample Preparation for Peptide and Protein Analysis One of the most important aspects of MALDI TOF-MS is the preparation of a *good* sample (5,6). It is not as simple as just applying a matrix material (Table 1) and an analyte to the sample support surface. Many variables influence the integrity of a good, homogeneous MALDI sample and may include the concentration of the matrix and analyte, choice of matrix, analyte sample history (i.e.,exposure to strong ionic detergents, formic acid), hydrophobicity or hydrophilicity character, contaminants and compatible solubilities of matrix and analyte solutions, just to mention a few.

Table 1: Some common MALDI matrices at 337 nm.

Matrix	*Application*
2,5-Dihydroxybenzoic acid (DHB)	Peptides, proteins, lipids, and oligosaccarides
3,5-Dimethoxy-4-hydroxycinnamic acid (sinapinic acid)	Peptides, proteins, and glycoproteins
a-Cyano-4-hydroxycinnamic acid (CHCA)	Peptides, proteins, lipids, and oligonucleotides

For a typical MALDI analysis, the peptide/protein (about 10 µM) and matrix (about 10 mM) solutions can be premixed in a small Eppendorf tube or applied directly to the sample support (i.e., sample plate). It is important that neither the matrix nor the analyte precipitates when the two solutions are mixed. Once the sample is applied to the sample support, the sample is allowed to air evaporate. Do not heat the sample to increase the evaporation rate. Changing the temperature alters the crystal growth and protein incorporation, which usually results in a poor MALDI sample. The dried sample is quite stable and can be stored at room temperature in the dark or in a vacuum for several days or more. Other desirable practices for producing good MALDI samples include the use of fresh matrix solutions whenever possible. Keep the crystal-growing "mother liquor" on the sample support below pH 4 so as not to ionize the matrix. Using 0.1% TFA will usually eliminate any adverse effects caused by a pH above 4. Do not use nonvolatile solvents because they can interfere with the crystal growth. Keep the concentration of non-protein material to a minimum so the ion signal is not suppressed.

There may be an occasion where poor MALDI mass spectra are produced even though the analyst believes the sample is of good MALDI quality (and the instrument is performing perfectly). If there is a known (or suspected) high level of contaminant (Table 2) that may be problematic with a MALDI analysis, the dried crystalline MALDI sample may be washed with 5-10 µl of distilled deionized water (or 0.1% TFA), provided the matrix is not highly water-soluble. (The wash step may be repeated as necessary.) This additional preparation step can sometimes produce a high quality MALDI sample, which is required for a successful analysis. The cinnamic acid derivative matrices are good matrices to use if a wash cycle is necessary because these matrices are only slightly water-soluble whereas DHB is readily soluble in water. A different MALDI matrix can be used since the choice of matrix can greatly effect the MALDI results in some cases. It should not be surprising that not all samples work well in every MALDI matrix since each peptide/protein has a unique structure that needs to be incorporated into a specific matrix crystal lattice. Also important to note is that each matrix compound has its own unique physical properties and interacts with the analyte molecules in a unique manner. If difficulty in getting adequate quality mass spectra still arises, or the quantity of analyte is small or the analyte solutions are very dilute, the fast-evaporation sample preparation (8) (or other sample preparation protocols) may be worthwhile trying. This sample preparation also works well for very large proteins (>100 kDa).

Table 2: Contaminant Concentration Tolerated in MALDI -TOF-MS (7).

Contaminant	Maximum allowable concentration (approx.)
Urea	0.5M
Guanidine-HCl	0.5M
Dithiothreitol	0.5M
Glycerol	1%
Alkali metal salts	<0.5M
Tris buffer	0.05M
NH_4HCO_3	0.05M
Phosphate buffer	0.01M
Detergents (not SDS)	0.1%
SDS	0.01%

Sequencing with MALDI TOF-MS

Introduction Historically, both linear and reflectron MALDI-TOF-MS have been utilized primarily for molecular weight determinations of molecular ions and enzymatic digests leading to structural information of proteins. These digests are typically mass analyzed with or without purification prior to molecular weight determinations. Varieties of methodologies have been developed to obtain primary sequence information for proteins and peptides utilizing MALDI TOF-MS. Two different approaches can be taken. The first method is known as protein ladder sequencing and is employed to produce structurally informative fragments of the analyte prior to insertion into the TOF mass spectrometer and subsequent analysis. The second approach utilizes the phenomenon of metastable ion decay that occurs inside the TOF mass spectrometer to produce sequence information. The labeling of the fragment ions shown in the following mass spectra uses the generally accepted nomenclature developed by Biemann (9) (Figure 4).

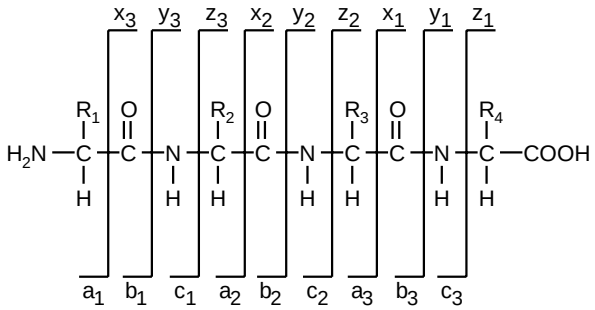

Figure 4. Illustration of the fragments formed through bond cleavages along the backbone of protonated linear peptides.

Ladder Sequencing with TOF-MS Proteins/peptides can be sequenced using MALDI -TOF-MS with a ladder sequencing technique (10) which consists of either a time-dependent or concentration-dependent chemical degradation from either the N- or C-terminus of the protein/peptide into fragments, each of which differs by one amino acid residue. The mixture is mass analyzed in a single MALDI -TOF-MS experiment with mass differences between adjacent mass spectral peaks corresponding to a specific amino acid residue. This type of analysis can be thought of as simply determining the masses of a series of peptides/proteins that are present in a single MALDI sample. The order of occurrence in the mass spectrum defines the sequence of amino acids in the original protein/peptide.

Shown in Figure 5 are the results of a ladder sequencing experiment utilizing the carboxypeptidase Y (CPY) concentration-dependent technique and linear MALDI-TOF-MS employing DE. The CPY digestion of adrenocorticotropic hormone (ACTH) fragment 18-39 (2465.70 Da) was performed directly on the MALDI sample support by adding 1 pmol of peptide to each of five sample wells and varying the concentration of CPY that was added to these applied peptides samples. The total time required to carry out this digest and prepare the samples for MALDI analysis was less than 15 minutes. The samples were inserted into the TOF mass spectrometer and the masses of the truncated (i.e., ladders) peptides were determined. The sequence of the peptide from the C-terminus can be read directly from the mass spectrum by determining the mass difference between adjacent peaks.

Post-Source Decay with RETOF-MS MALDI has historically been considered a "soft" ionization technique that produces almost exclusively intact protonated pseudomolecular ion species. Studies (11) have shown that a significant degree of metastable ion decay occurs after ion acceleration and prior to detection. The ion fragments produced from the metastable ion decay of peptides and proteins typically include both neutral molecule losses (such as water, ammonia and portions of the amino acid side chains) and random cleavage at peptide bonds.

The observance of these metastable ion decay products in MALDI mass spectra is dependent on the TOF instrumental configuration. For any MALDI experiment employing TOF mass spectrometers with continuous ion extraction, metastable ion decay occurring in the ion source during ion acceleration produces incoherent ion noise in the resulting mass spectrum. In a linear TOF mass spectrometer, metastable fragmentation which occurs post-source (i.e., in the field free region) is not apparent since precursor and metastable decay ions move with the same velocity (provided there are no post-source electrostatic fields) and arrive simultaneously at the detector. Conversely, a RETOF mass spectrometer will separate precursor and metastable decay ions by their difference in kinetic energy in the ion mirror. The majority of metastable ion decay observed in RETOF mass spectrometers occurs during ion acceleration out of the ion source and in the field free region of the mass spectrometer (i.e., post-source decay). Recent approaches utilizing the post-source decay (PSD) fragmentation of the metastable ions produced with MALDI in a RETOF mass spectrometer has been reported by

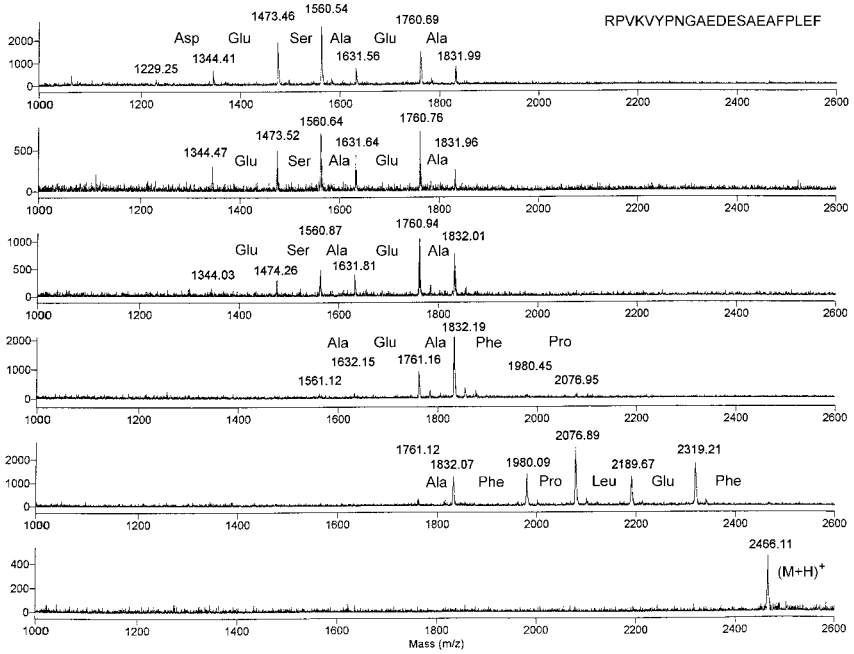

Figure 5. Ladder sequencing of the adrenocorticotropic hormone (ACTH) fragment 18-39 utilizing the CPY dilution technique and MALDI linear DE-TOF-MS. 25 kV accelerating potential, 150 ns pulse delay, 1.30 kV pulse, 50 laser pulses averaged for each spectrum.

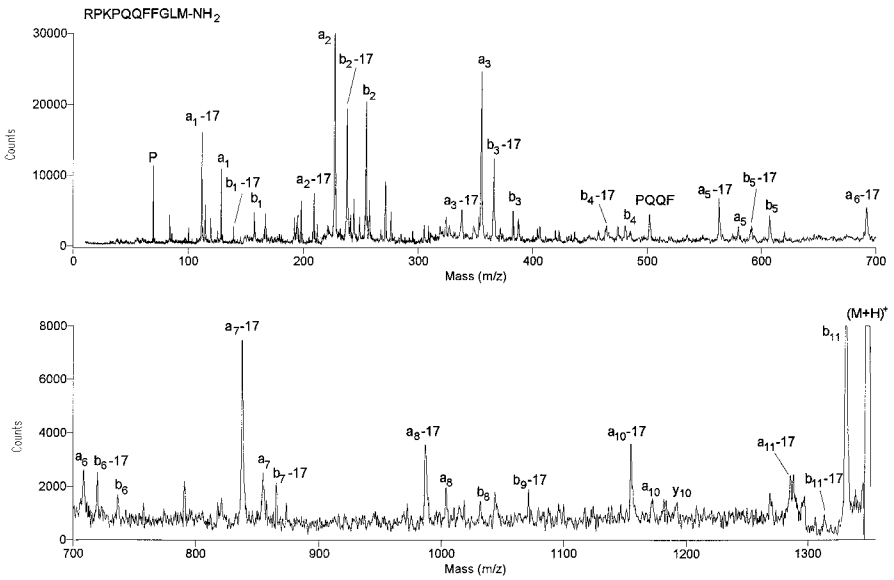

Figure 6. PSD mass spectrum of substance P in CHCA employing RE-TOF-MS. 15 kV accelerating potential, 50 laser pulses averaged for each reflector voltage segment.

Spengler (12). In the PSD technique, some fraction of the MALDI generated ions undergo metastable decay during flight by either unimolecular or bimolecular (collisions) pathways producing smaller m/z ions and neutrals. Although only a single kinetic energy value is perfectly focused at a specific reflectron potential, approximately 5-10% of the entire kinetic energy range of the fragment ions can be adequately focused at this potential to produce a portion of the entire mass spectrum. This requires the reflectron potential to be systematically stepped in order to bring other m/z ions with different kinetic energies into focus. A composite mass spectrum can be produced by combining the individual portions of the mass spectrum that were produced from the 10-20 different reflector potential steps that were required for observing the entire mass spectrum of fragment ions containing the desired sequence information.

The fragmentation pattern in PSD-RETOF favors backbone cleavages producing predominantly a_n, b_n and y_n type fragment ions with very little (if any) side chain specific cleavages. Another common occurence in PSD-RETOF mass spectra is the predominance of fragment ions less 17 Da which is usually thought to be due to the loss of ammonia from the N-terminal residue. The representative PSD mass spectrum of substance P (1347.65 Da) is shown in Figure 6.

Interpretation of PSD mass spectra takes time and practice in order to become proficient. It should be remembered that the fragmentation observed is residue specific. A systematic approach for analyzing PSD data should include the identification (if possible) of amino acid immonium (H_2N^+=CHR) ions and internal acyl ions (b_n like ions) as well as the starting N- and/or C-termini amino acid chains. Look for mass differences between peaks especially those with differences of 28u (b_n - a_n) and doublets with a mass difference of 17u (eg., a_n and a_n-17). Combine the above information to deduce the primary sequence. If there is not enough obtainable sequence information from the PSD data, then derivatization procedures for the analyte with subsequent mass spectral analyses may be necessary to obtain the desired information.

In-Source Decay with Linear TOF-MS An alternative approach to RETOF-MS for studying metastable ion decay of MALDI generated ions is to utilize DE with linear TOF-MS (13). By employing the DE technique, primary structural information for peptides and proteins can also be obtained. Prompt ion fragmentation produced at the time of the desorption event (i.e., ion formation) is generally absent for MALDI generated peptide/protein ions. By incorporating a time delay between ion formation and ion extraction, ions in the source are allowed to fragment in a relatively short period of time (<100 ns) into smaller ions and neutrals prior to extraction. A drawout potential is then applied extracting the fragmented ions. Coherent mass spectral peaks are produced from these metastable decayed ions giving rise to significant structural information for peptides and proteins. The observed c_n, y_n and y_n less a net loss of 15 Da fragment ion series produced from the fast metastable ion decay process is much different than the fragmentation produced from the more widely used PSD MALDI technique which yields a_n, b_n and y_n fragment ion series. The masses of the y_n less a net loss of 15 Da fragment ions are equivalent to z_n+2 ions. For this reason, these y_n less a net loss of 15 Da ions will be referred to as z_n+2 ions although further studies are needed to determine if these ions are actually z_n+2 ions.

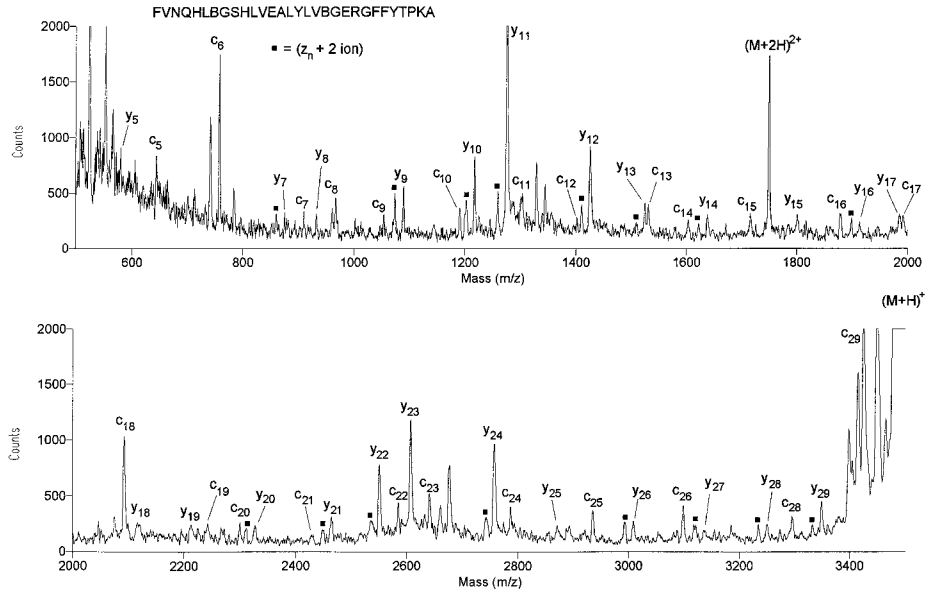

Figure 7. MALDI fast metastable ion fragmentation of the oxidized B chain of bovine insulin in 2,5-DHB employing linear DE-TOF-MS. 25 kV accelerating potential, 150 ns pulse delay, 1.45 kV pulse, 50 laser pulses averaged. Residue B represents an oxidized cysteine.

A major advantage of utilizing DE linear MALDI TOF-MS for the investigation of fast metastable ion fragmentation is the ease and speed of conducting an experiment. A single set of instrumental parameters can produce an analytically useful mass spectrum in less than a few minutes. The resulting mass spectrum comprises the entire mass range of metastable decay ions produced from peptide and small protein precursors. The obtainable mass resolution throughout the mass spectrum is also quite good (m/Δm typically ranges between 500 and 1000 for all ion signals observed) even though the DE instrumental parameters are typically optimized for the singly protonated pseudomolecular ion. Mass calibration of each DE linear TOF mass spectrum can utilize a simple two point calibration fitting a linear equation to the experimental flight times of two known m/z ions whose molecular weights have been calculated producing mass accuracies typically better than 0.5 Da in most cases.

A potential major application for the DE technique may lie in the area of sequencing chromatographically separated and isolated digest peptides, whereby significant amounts of structural information can be obtained from limited quantities of analyte. MALDI fast metastable ion decay is observed for the oxidized B chain of bovine insulin (3495.94 Da) in DHB (Figure 7). This fast metastable ion decay mass spectrum is dominated by c_n, y_n and z_n+2 fragment ions which provides a significant amount of sequence specific information for bovine insulin chain B. The fragmentation observed provides overlapping sequence information for the peptide from both the N terminus and C terminus. This information can be directly correlated to the known sequence for the oxidized B chain of bovine insulin. Fast metastable fragment ion peaks corresponding to fragmentation at each amino acid residue in the peptide are present in the fast metastable ion decay mass spectrum with the exception of the c_{27} ion fragment (loss of proline) and the low m/z ion fragments (c_1-c_4, y_1-y_4, y_6 and z_1+2-z_6+2). The lack of a c_{27} ion fragment associated with the loss of a proline residue is most likely due to the cyclical nature of the residue which results in a tertiary, instead of a secondary, amide. Fragmentation at a proline residue via c_n or z_n+2 fragmentation pathways would require the cleavage of two bonds in order to observe these fragments. This is not a very likely occurrence. A high abundance of low mass and matrix ion signals below a m/z of 400-500 (dependent upon the matrix employed) preclude observation of fast metastable fragment ions (if they are present) in this m/z region of the mass spectrum.

Interpretation of in-source fragmentation mass spectra should be approached in a manner similar to that taken with PSD interpretation. The limitation of this interpretation occurs in the low mass region of the mass spectrum where the abundance of intense, low mass matrix (and other) ion signals typically dominate the linear MALDI spectra and make interpretation difficult in this region. If insufficient information for sequence determination has resulted from the fragmentation analysis, changing the matrix may provide additional information since it has been observed (14) that fast metastable ion fragmentation is very much matrix dependent. From preliminary studies involving in-source fragmentation, it appears that DHB works best for peptides while sinapinic acid is preferred as the matrix for larger analytes in the small protein mass range.

Conclusion

MALDI TOF-MS has developed into a valuable tool in the biosciences for obtaining both accurate mass determinations and primary sequence information. The sequence information obtained from the mass spectra shown in this section whose sequence was known *a priori* by no means implies a straightforward scheme to deduce an unknown peptide or protein sequence from its metastable ion decay mass spectrum. These MALDI techniques are envisioned to be most useful in conjunction with conventional biochemical techniques such as protein digests. They should be applicable to identifying blocked amino termini, post-translational modifications and mutation sites in known proteins in this way. Also, with a total unknown, a significant amount of preliminary structure determination should be possible on very small (less than 10 pmol) amounts of analyte. For ladder sequencing and in-source fragmentation studies, it is important to minimize potential peptide impurities. Careful attention must also be given to synthetic peptide samples so as not to confuse the fragment ion signals with protonated molecular ions originating from low levels of incomplete synthesis impurities.

References

1. Karas, M., Bachmann, D., Bahr, U. and Hillenkamp, F., "Matrix-assisted ultraviolet laser desorption of non-volatile compounds". *Int. J. Mass Spectrom. Ion Process.* 1987, *78*, 53.
2. Ingendoh, A., Karas, M., Hillenkamp, F. and Giessmann, U., "Factors affecting the mass resolution in matrix-assisted laser desorption-ionization mass spectrometry". *Int. J. Mass Spectrom. Ion Process.* 1994, *131*, 345.
3. Brown, R. S. and Lennon, J. J., "Mass resolution improvement by incorporation of pulsed ion extraction in a matrix-assisted laser desorption/ionization linear time-of-flight mass spectrometer". *Anal. Chem.* 1995, *67*, 1998.
4. Vestal, M. L., Juhasz, P. and Martin, S. A., "Delayed extraction matrix-assisted laser desorption time-of-flight mass spectrometry ". *Rapid Comm. Mass Spectrom.* 1995, *9*, 1044.
5. Cohen, S. L. and Chait, B. T., "Influence of matrix solution conditions on the MADLI-MS analysis of peptides and proteins". *Anal. Chem.* 1996, *68*, 31.
6. Beavis, R.C. and Chait, B.T., "Matrix-assisted laser desorption ionization mass-spectrometry of proteins". *Methods in Enzymol*. 1996, *270*, 519.
7. Coligan, J. E., Dunn, B. M., Ploegh, H. L., "Matrix-assisted laser desorption/ionization time-of-flight mass analysis of peptides". Speicher, D. W. and Wingfield, P.T. Eds. (Contributed by William J. Henzel and John T. Stults), *Current Protocols in Protein Science, Volume 1*, John Wiley & Sons, New York, N. Y., 1995, Unit 16.2.
8. Vorm, O., Roepstorff, P. and Mann, M., "Improved resolution very high sensitivity in MALDI TOF of matrix surfaces made by fast evaporation". *Anal. Chem.* 1994, *66*, 3281.
9. Biemann, K., "Nomenclature for peptide fragment ions (positive ions)", in *Mass Spectrometry*, McCloskey, J.A., Ed., Methods in Enzymology 193, Academic Press, San Diego, 1990, Appendix 5, pp. 886-7.
10. Patterson, D. H., Tarr, G. E., Regnier, F. E. and Martin, S. A., "C-terminal ladder sequencing via matrix-assisted laser desorption mass spectrometry coupled with carboxypeptidase Y time-dependent and concentration-dependent digestions". *Anal. Chem.* 1995, *67*, 3971.
11. Spengler, B., Kirsch, D. and Kaufmann, R., "Metastable decay of peptides and proteins in matrix-assisted laser-desorption mass spectrometry". *Rapid Commun. Mass Spectrom.* 1991, *5*, 198.
12. Spengler, B. "New Instrumental Approaches to Collision-Induced Dissociation Using a Time-of-Flight Instrument" in *Peptide and Protein Analysis by Mass Spectrometry*, Chapman, J. R., Ed., Methods in Molecular Biology 61, Humana Press, Totowa, N. J., 1996, 43.
13. Brown, R. S. and Lennon, J. J., "Sequence-specific fragmentation of matrix-assisted laser-desorbed protein/peptide ions". *Anal. Chem.* 1995, *67*, 3990.
14. Brown, R. S., Carr, B. L. and Lennon, J. J., "Factors that influence the observed fast fragmentation of peptides in matrix-assisted laser desorption". *J. Am. Soc. Mass Spectrom.* 1996, *7*, 225.

John Lennon can be contacted at the Department of Biochemistry, Box 357350, University of Washington, Seattle, WA 98195, Tel: (206) 543-7495, Fax: (206) 685-1792, E-mail: jjl@u.washington.edu.

Mission Statement
from the Bylaws of the Association
of Biomolecular Resource Facilities

The ASSOCIATION OF BIOMOLECULAR RESOURCE FACILITIES has been organized for the following purposes:

A. To promote and support resource facilities, research laboratories, and individual researchers regarding operation, research, and development in the areas of methods, techniques, and instrumentation relevant to the analysis and synthesis of biomolecules.

B. To provide mechanisms for the self-evaluation and improvement of procedural and operational accuracy, precision, and efficiency in resource facilities and research laboratories.

C. To provide a mechanism for the education of resource facility and research laboratory staff, users, administrators, and interested members of the scientific community.